REMARKS

Applicants and their Attorney thank the Examiner and Examiner Naff for the courtesy of the November 15, 2007 telephonic interviews during which the outstanding rejections were discussed.

Claims 1-52 were pending in the instant application. Claims 1, 5, 6 and 52 have been amended. Support for the amendments to claims 1, 5, 6 and 52 can be found in the specification and claims as originally filed. Specifically, support for the amendments to claims 1, 5, 6 and 52 can be found at least at page 18, line 32 through page 19, line 32 of the specification. Upon entry of the present Amendment, claims 1-52 are pending and presented for reconsideration. Applicants respectfully submit that no new matter is introduced by the present Amendment.

Amendment and/or cancellation of the claims is not to be construed as acquiescence to any of the objections/rejections set forth in the instant Office Action or any previous Office Action of the parent application, and was done solely to expedite prosecution of the application. Applicants submit that claims were not added or amended during the prosecution of the instant application for reasons related to patentability. Applicants reserve the right to pursue the claims, as originally filed, or similar claims in this or one or more subsequent patent applications.

Rejection of Claims 1-6 and 52 under 35 U.S.C. §112, First Paragraph

Claims 1-6 and 52 have been rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. In particular, the Office Action, on page 5, states that "[t]he specification as currently presented while describing the treatment of a microbial infection via administering a modulator of a transcription factor to an individual in need thereof does not provide support for a method to prevent said infecting in an individual." Applicants traverse the foregoing rejection on the grounds that one of ordinary skill in the art would understand that Applicants were in possession of the claimed invention.

The pending claims are directed to a method for an preventing infection, *e.g.,* prostatitis or urinary tract infection, of a subject by a microbe comprising: administering a compound that downmodulates the expression or activity of a microbial transcription factor to a subject at risk of developing an infection, wherein the downmodulation of the microbial transcription factor reduces the virulence of the microbe, such that infection is prevented. For the reasons set forth

below, Applicants respectfully submit that the claimed invention is supported by the specification on record and that the instant specification conveys to the ordinary skilled artisan that the inventor(s) had possession of the claimed invention at the time the application was filed.

An objective standard for determining compliance with the written description requirement under 35 U.S.C. § 112, first paragraph, is whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, the applicant was in possession of the invention as now claimed. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991) and *In re Gosteli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989).

To begin with, the Examiner has admitted, at page 5 of the Office Action, that the specification describes "the treatment of a microbial infection via administering a modulator of a transcription factor to an individual in need thereof." Moreover, Applicants respectfully submit that Applicants' specification also provides extensive teachings on methods for preventing infection of a subject by a microbe comprising: administering a compound that downmodulates the expression or activity of a microbial transcription factor to a subject at risk of developing an infection, wherein the downmodulation of the microbial transcription factor reduces the virulence of the microbe, such that infection is prevented.

The Data Presented in the Application Demonstrates ***Prevention*** of Kidney Infection

In the application Applicants teach an animal model of kidney infection, the ascending pyelonephritis model. In this *in vivo* infection model, bacteria are introduced into the ***bladder*** and their ability to colonize the ***kidneys*** is measured. Accordingly, bacteria are introduced into the bladder of the mouse and the mice are concurrently with a dose of inhibitor compound. After a designated period of time, the mice are sacrificed and their kidneys removed and tested for the presence of bacteria.

The model can be used to test, *e.g.,* whether a particular strain of bacteria can migrate to the kidneys and establish an infection there or whether a particular test compound can prevent infection of the kidney by a strain of bacteria. Accordingly, by dosing the mice with inhibitor compound at the time of bacterial inoculation into the bladder, Applicants can test whether ***infection of the kidneys is prevented***.

For Example, the ability of test compounds to prevent infection of the kidneys after bacteria have been introduced into the bladder is measured. In this Example, Applicants teach

that mice were treated once, at the time of infection (which is before any colonization of the kidney) and treatment with a dose of 100 mg/kg of inhibitor prevented kidney infection in 100% of the mice tested, *e.g.,* 0 out of 5 mice were infected (see, *e.g.,* the chart on page 127 of the specification). Furthermore, Applicants further teach that lower doses of inhibitor, *e.g.,* 10 mg/kg and 1 mg/kg, can also prevent infection in a substantial percentage of the mice (see, *e.g.,* the chart on page 127 of the specification). In Example 12 on page 132 of the specification, the efficacy of one prototypic inhibitor was investigated using the ascending pyelonephritis model. The administration of a single subcutaneous dose of the inhibitor at the time of infection was sufficient to prevent infection in this *in vivo* model (see Figure 10 of the specification). Furthermore, the specification teaches at page 132, lines 29-33, that "[r]esults similar to those obtained with the single 100 mg/kg dose (Fig. 10) were observed using smaller doses with multiple dose regimens (bid x4 d, data not shown)." Accordingly, administration of a microbial transcription factor prior to kidney infection (at the time of inoculation of the bladder with bacteria) can prevent infection of the kidneys.

Microbial Transcription Factors Have Been Validated as Targets

As taught in the instant application, if a MarA family transcription factor gene is knocked-out in a bacterial strain, the bacterial strain is rendered avirulent and any subsequent bacterial infection is prevented. Thus, ***knocking out microbial transcription factor genes or inhibiting of transcription factor activity renders bacteria avirulent and prevents the establishment of infection***. These data show that deletion of rob or soxS alone is sufficient to confer the avirulent phenotype on organisms. Moreover, supplying either rob or soxS in their original chromosomal locations in either the single (PC1037 and PC1038) or triple (SRM) knockout backgrounds fully restored virulence in these strains. With respect to marA, when marA is supplied in its original chromosomal location in the triple knockout background (PC1012), virulence is fully restored. Thus, these transcription factors can be considered to be virulence factors and preventing their function should prevent the ability of organisms to establish infection.

Inhibitors of Microbial Transcription Factors Do Not Have Intrinsic Antibacterial Activity

The compounds of the invention have no intrinsic antibacterial activity (see, *e.g.,* page 37 of the application, third full paragraph). These inhibitory compounds do not affect the bacteria's ability to grow and have no direct effect on the bacteria other than preventing the initial infectious process. Accordingly, the compounds of the invention downmodulate the expression or activity of a microbial transcription factor, thereby preventing establishment of infection.

Applicant's results have been published in a peer-reviewed Journal, attached hereto as Appendix A; Casaz *et al.,* Microbiology, 152:3642-50 (2006).

In view of the foregoing, one of ordinary skill in the art would understand that Applicants were in possession of the claimed invention at the time of filing of the instant application. Applicants, therefore, respectfully request withdrawal of the rejection of claims 1-6 and 52 under 35 U.S.C. §112, first paragraph and favorable reconsideration.

CONCLUSION

In view of the above amendments and remarks, Applicants believe that the pending application is in condition for allowance. Applicants believe that no fee is due with this communication. However, if a fee is due, please charge our Deposit Account No. 12-0080, under Order No. PAZ-190RCE from which the undersigned is authorized to draw.

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Respectfully submitted,

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Appendix A

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MarA, SoxS and Rob function as virulence factors in an *Escherichia coli* murine model of ascending pyelonephritis

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MarA, SoxS and Rob are transcription factors belonging to the AraC family. While these proteins have been associated historically with control of multiple antibiotic resistance, and tolerance to oxidative stress agents and organic solvents, only a paucity of experimental data support a role in regulating virulence. Clinical *Escherichia coli* isolates, and isogenic strains lacking *marA*, *soxS* and *rob*, were studied in a murine model of ascending pyelonephritis, which is a clinically relevant model of urinary tract infection. Organisms lacking all three transcription factors (triple knockouts) were significantly less virulent than parental strains, and complementation studies demonstrated that the addition of *marA*, *soxS* and *rob* individually restored wild-type virulence in the triple-knockout strain. Deletion of *soxS* or *rob* alone was more detrimental than the removal of *marA*. Thus, all three proteins contribute to virulence *in vivo*.

INTRODUCTION

The AraC family of transcription factors is composed of more than 1000 members (Alekshun & Levy, 2004), many of which have well-known roles as virulence factors (Finlay & Falkow, 1997). ExsA from *Pseudomonas aeruginosa* regulates a type III secretion system (TTSS) (Hauser *et al.*, 1998), *Yersinia* spp. LcrF (VirF) and YbtA control a TTSS (Flashner *et al.*, 2004) and yersiniabactin (siderophore) (Fetherston *et al.*, 1996) biosynthesis, respectively, and ToxT from *Vibrio cholerae* governs the synthesis of cholera toxin and toxin co-regulated pili (Champion *et al.*, 1997). Inactivation of genes specifying AraC family members [e.g. BfpT, ToxT, LcrF (VirF), Rv1931c, ExsA, Sp1433 and MarA] attenuates virulence in human subjects (Bieber *et al.*, 1998) and a variety of animal infection models (Champion *et al.*, 1997; Flashner *et al.*, 2004; Frota *et al.*, 2004; Hauser *et al.*, 1998; Hava & Camilli, 2002; Randall & Woodward, 2001).

Thus, in addition to a primary role in virulence, it is assumed that many members of the AraC family play larger roles in affecting the overall physiology of the bacterial cell. Notably, genomic array experiments have shown that *P. aeruginosa* ExsA and *V. cholerae* ToxT regulate the expression of a large collection of genes termed regulons (Bina *et al.*, 2003; Wolfgang *et al.*, 2003).

Escherichia coli MarA and SoxS were originally identified based on their ability to control multiple antibiotic resistance (Mar) (George & Levy, 1983a, b), and susceptibility to superoxide and other oxidative stress agents (Wu & Weiss, 1991), respectively. Experiments with Rob, a MarA and SoxS paralogue, showed that it could function in a similar manner (Ariza *et al.*, 1995). Subsequent data have documented multidrug-resistant clinical strains of *E. coli* (Linde *et al.*, 2000; Maneewannakul & Levy, 1996) and *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) (Koutsolioutsou *et al.*, 2001) that constitutively express AraC family members. It is therefore surmised that these proteins may play a role in the infectious process.

Although the *soxS* and *mar* loci are expressed by *S. typhimurium* within macrophages (Valdivia & Falkow, 1996) and J774-A.1 (macrophage-like) cells (Eriksson *et al.*, 2003), initial attempts to demonstrate experimentally an effect on virulence *in vivo* have been unsuccessful. Sulavik *et al.* (1997) used a lethal infection model to investigate the virulence of strains lacking *soxS*. Van der Straaten and colleagues (2004) failed to find differences during *in vivo* growth of wild-type organisms compared with strains lacking *ramA* (specifying another AraC protein), *soxS* or

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Abbreviations: TTSS, type III secretion system; UPEC, uropathogenic *E. coli*; UTI, urinary tract infection; VUR, vesicoureteral reflux.

both genes. Randall & Woodward (2001), however, found that *S. typhimurium* DT104 lacking *marA* was less likely to colonize the spleens and caeca of infected chicks.

MarA, SoxS and Rob each regulate the expression of multiple genes (called the MarA, SoxS and Rob regulons) in *E. coli* (Barbosa & Levy, 2000; Bennik *et al.*, 2000; Pomposiello *et al.*, 2001), *S. typhimurium* (Pomposiello & Demple, 2000) and, by inference, other members of the *Enterobacteriaceae.* This regulation is achieved by the binding of the transcription factor to a degenerate sequence, called the 'marbox' or 'soxbox' (Martin & Rosner, 2003), which is located within the promoter of the regulated gene. Since MarA has been found recently to act as a transcription repressor (Schneiders *et al.*, 2003), it is more precise to state that the location of the marbox can be found among both promoter and operator sequences. The degeneracy of the marbox/soxbox allows for the differential regulation of individual genes by MarA, SoxS and Rob (Martin *et al.*, 2000; Martin & Rosner, 2003). Thus, experiments investigating phenotypes attributed to these proteins should be performed with strains lacking single and multiple members of this transcription factor family.

Using a murine model of pyelonephritis, we found that *E. coli* lacking *marA*, *soxS* and *rob* was unable to maintain colonization of the kidney. In this model, the bladders of diuresed mice were infected with bacteria. Subsequently, the organisms migrated to the kidneys in a process that mimics the clinical course of pyelonephritis. When the individual loci were restored in single copies, each was capable of restoring wild-type virulence.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* KM-D is a clinical isolate obtained from an intestinal fistula, and bears a mutated *marR*, resulting in a Mar phenotype (Maneewannakul & Levy, 1996). *E. coli* C189 is a clinical cystitis isolate that is multidrug

Table 1. Bacterial strains and plasmids used in this study

Strain (serotype if relevant) or plasmid	Relevant characteristics/genotype	Source or reference
***E. coli* strains**		
S17-1λ*pir*	*lamB* F⁻ *supE44 thi-1 thr-1 leuB6 lacY1 tonA21 hsdR hsdM recA pro* (RP4:2-Tc::Mu::Km::Tn*7*) λ *pir*	Simon *et al.* (1983)
DH5αλ*pir*	F⁻ Φ*80 lacZ*ΔM15 *endA1 recA1 hsdR17*(r_k^- m_k^-) *supE44 thi1 gyrA96 relA1*Δ(*lacZYA-argF*) *U169* λ *pir*	Elliott & Kaper (1997)
MG1655	K-12 laboratory isolate	In-house strain collection
EP-1	Clinical isolate	Linde *et al.* (2000)
KM-D	Clinical isolate, *marR*	Intestinal fistula; Maneewannakul & Levy (1996)
C189 (O2:H⁻)	Clinical isolate	Cystitis; Yamamoto *et al.* (1995)
PC1001	KM-D Δ*marA*	This study
PC1003	KM-D Δ*rob*	This study
PC1005	KM-D Δ*soxS*	This study
PC1012	KM-D Δ*soxS, rob, marA*	This study
PC1031	PC1001::*marA*	This study
PC1033	PC1012::*marA*	This study
PC1035	PC1005::*soxS*	This study
PC1037	PC1012::*soxS*	This study
PC1038	PC1012::*rob*	This study
PC1040	PC1003::*rob*	This study
C189-P2	C189 passaged twice in mice	This study
PC*marA*	C189-P2, *marA*	This study
PC*soxS*	C189-P2, *soxS*	This study
PC*rob*	C189-P2, *rob*	This study
PC*rob, soxS*	C189-P2, *rob, soxS*	This study
PC wt Rob	C189	This study
PC wt MarA	C189	This study
Plasmids		
pSR47s	Km^R R6K*oriV* RP4*oriT sacB*	Kolter *et al.* (1978)
pPCΔ*rob*	pSR47s with DNA sequences flanking *rob*	This study
pPCΔ*soxS*	pSR47s with DNA sequences flanking *soxS*	This study
pPCΔ*marA*	pSR47s with DNA sequences flanking *marA*	This study

susceptible (Rippere-Lampe *et al.*, 2001; Yamamoto *et al.*, 1995). *E. coli* C189-P2 was obtained following two passages of *E. coli* C189 in mice of the infection model described below; there was no change in drug susceptibility in the derived strain C189-P2.

Genetic techniques. In-frame (non-polar) deletions of specific genes in KM-D or C189-P2 were constructed by crossover PCR and allelic exchange (Link *et al.*, 1997). A 1 kb DNA fragment consisting of 500 bp flanking the upstream and downstream portions of the sequences targeted for deletion, separated by a 33 nt spacer, was constructed by crossover PCR, and cloned into the *NotI–BamHI* site of the suicide vector pSR47s. pSR47s contains the R6K origin of replication, rendering it dependent on the π protein, the kanamycin-resistance gene from Tn*903*, and the *Bacillus subtilis sacB* gene, which is used as a counterselectable marker (Kolter *et al.*, 1978). Plasmids with the cloned crossover PCR fragments were transferred from *E. coli* S17-1λ*pir* to KM-D and C189-P2 by conjugation, and transconjugants were selected on M9 minimal medium containing 0·2% glucose and 30 μg kanamycin ml^{-1}. KM-D and C189-P2 transconjugants were then grown overnight at 37 °C in Luria–Bertani (LB) without antibiotics. The overnight cultures were diluted in double-distilled water, and 10^5–10^6 c.f.u. were plated on L agar containing 5% sucrose, and incubated at 30 °C overnight. The resulting colonies were plated on LB agar with and without kanamycin. Kanamycin-sensitive colonies were tested for the presence or absence of the wild-type and deleted alleles by PCR with allele-specific primers.

The crossover PCR products used for the in-frame deletion have a 33 nt 'stuffer' sequence containing a *SpeI* restriction site. In order to restore the deleted genes into their original loci, the wild-type genes were amplified from KM-D and C189-P2 colonies with primers that created *SpeI* restriction sites at both ends of the ORF. These fragments were restricted with *SpeI*, and ligated to the plasmids used to make the corresponding in-frame deletions. This procedure recreates the original gene, with an additional seven amino acids (Met-Val-Ile-Asn-Leu-Thr-Gly) at the amino terminus. This complementation plasmid was recombined into the chromosome of the appropriate mutant strains by allelic exchange, as described above, and the presence of the wild-type allele was confirmed by PCR.

PCR was used to identify genes specifying virulence factors known to play a role in *E. coli* urinary tract infection (UTI; i.e. cystitis and pyelonephritis). Primers were designed, and PCR was performed as described by others (Ruiz *et al.*, 2002, and references therein) using total DNA isolated from C189-P2 and KM-D.

Assay for type I fimbriae expression. The assay for type I fimbriae expression was based on the protocol of Bahrani-Mougeot *et al.* (2002). Bacteria were grown in static LB broth for 48 h at 37 °C, centrifuged for 1 min, and resuspended in 1 ml PBS. A 25 μl aliquot was then added to a flat-bottom 96-well plate. Defibrinated guinea pig blood (Colorado Serum) was centrifuged at 2300 RCF (5000 r.p.m. in an IEC Micromax microcentrifuge) for 1 min, and washed once with PBS. The washed red blood cells were then diluted in PBS with or without 50 mM mannose, and 25 μl of this suspension was placed into the wells of a 96-well plate containing bacteria. The plate was rocked at room temperature for 15–20 min, and agglutination of the red blood cells was assessed by visual inspection of the wells, and at a magnification of ×100 under an inverted microscope. *E. coli* EP-1 (Linde *et al.*, 2000), which lacks the genes specifying type I fimbriae, as determined using PCR, was used as a negative control.

Ascending UTI model. All animal experiments were approved by our Institutional Animal Care and Use Committee. To make *E. coli* C189 suitable for studies in mice, it was passed twice through the murine host, as described below, and the resulting strain was designated C189-P2, to designate two *in vivo* passages. *E. coli* KM-D did not require these steps. The murine model of ascending pyelonephritis has been described by Hopkins *et al.* (1998). Briefly, CD-1 female mice (mean weight 20–30 g) were diuresed (diuresis was necessary for consistent kidney infections) on a diet consisting of water containing 5% glucose and restricted solid food (10–12 g per cage of five animals). On the day of the experiment, each mouse was anaesthetized with isoflurane, and the abdominal area was shaved and bathed with iodine, followed by sterilization with 2-propanol. A small incision was made through the outermost skin layer just above the urethra. Once the inner skin layer was exposed, another incision was made through the peritoneum, exposing the inner cavity and the bladder. A small puncture was made in the bladder to aspirate excess urine, and the infectious dose (100 μl) was introduced by intravesicular inoculation.

In order to investigate the growth conditions necessary to achieve reproducible kidney infections in the UTI model, mice were infected with *E. coli* KM-D grown under different conditions *in vitro*. Bacteria grown overnight in LB broth produced the most robust and reproducible infections. From an overnight culture grown at 37 °C in LB medium, bacteria were washed with PBS, diluted to 10^8 c.f.u. ml^{-1} in PBS, and 100 μl of this culture (10^7 c.f.u.) was used to inoculate the mice.

After a designated period of infection, mice were killed, and their kidneys were removed. Individual kidneys were weighed, and then suspended in 5 ml sterile PBS. The kidneys were homogenized, and 1 : 10 serial dilutions were plated on MacConkey agar to determine the infectious load (c.f.u.) per gram of kidney. All data are presented as median $\log_{10}$ c.f.u. (g kidney)$^{-1}$.

Statistical analyses. The statistical significance of differences between bacterial isolates was determined using ANOVA with normally distributed data, and with Dunnett's post-hoc tests. When no bacteria were recovered at a 10^{-2} dilution, a value of 1 was assigned to the c.f.u. (g kidney)$^{-1}$ in order to apply statistics using parametric models. P values of $<0{\cdot}05$ were considered significant.

RESULTS AND DISCUSSION

Virulence of wild-type and mutant bacteria

To investigate defects in virulence among bacterial strains lacking MarA, SoxS and/or Rob, we used a mouse model of ascending pyelonephritis. Since this model permits a determination of c.f.u. (g kidney)$^{-1}$, it enabled us to make quantitative assessments of bacterial load.

Since MarA, SoxS and Rob can regulate similar genes, there is redundancy among their regulons (see above). Therefore, to mitigate potential compensatory changes, we began our experiments with a strain deleted of all three genes. Both KM-D (parent) and PC1012 (bearing in-frame deletions in *marA*, *soxS* and *rob*) were cultured from the kidneys of mice for up to 3 days post-infection (Fig. 1a, b). After this period, KM-D maintained colonization of the kidneys for at least 11 days post-infection, while PC1012 was cleared from the kidney after day 3 (Fig. 1b); this difference was statistically significant ($P<0{\cdot}01$). These data suggest that the ability of PC1012 to reach the kidney (from the bladder) is not compromised, but that this strain is defective in its ability to maintain colonization of the kidney once there.

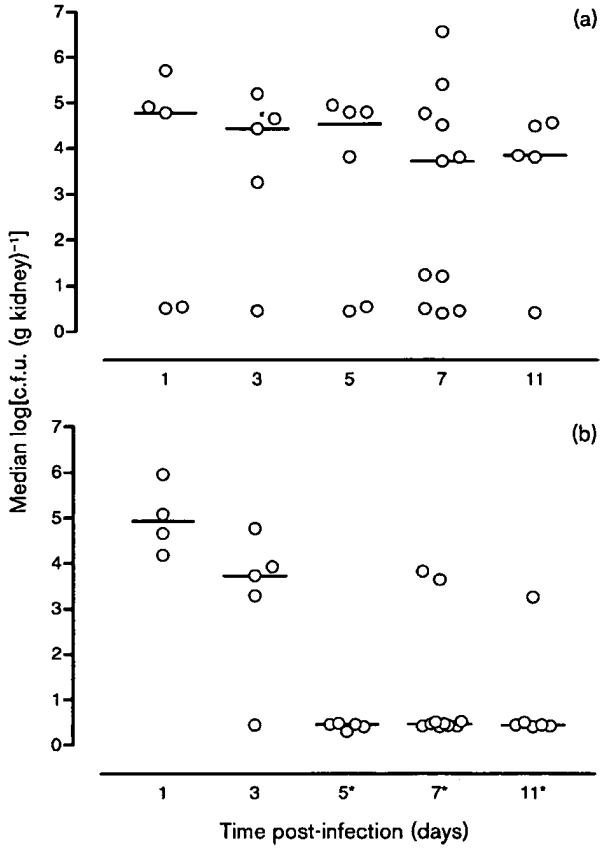

Fig. 1. Time course of kidney colonization in the mouse UTI model. Mice were infected via intravesicular inoculation (see Methods), kidneys were harvested, and bacterial loads were determined at the designated time points. (a) *E. coli* KM-D; no significant difference ($P>0{\cdot}05$) for day 1 versus days 3, 5, 7 and 11. (b) *E. coli* PC1012 (triple deletion); $^{*}P<0{\cdot}01$ for day 1 versus days 5, 7 and 11.

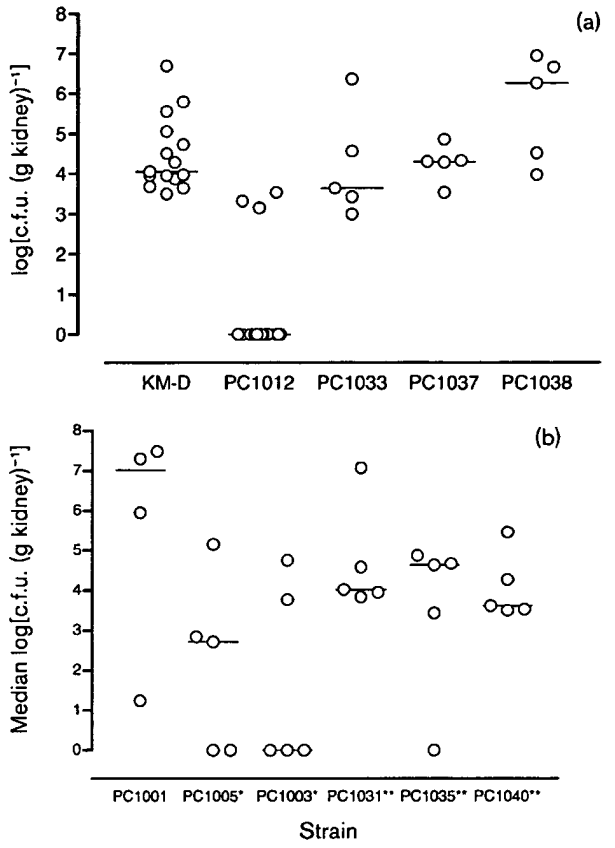

Fig. 2. Effects of transcription factor deletion on *E. coli* colonization in the KM-D genetic background in an ascending murine model of pyelonephritis. Mice were infected (see Fig. 1 legend), and kidneys were harvested at 5 days post-infection. (a) Complementation of the triple deletion (PC1012) strain; $^{*}P<0{\cdot}05$ versus KM-D with *marA*, *soxS* or *rob*. The data for KM-D and PC1012 were pooled from three separate experiments. (b) Comparison of single-deletion strains [PC1001 (Δ*marA*), PC1005 (Δ*soxS*) and PC1003 (Δ*rob*); $^{*}P\leqslant 0{\cdot}05$ versus KM-D; Fig. 2a], and complementation of strains deleted for a single transcription factor [PC1031 (+*marA*), PC1035 (+*soxS*) and PC1040 (+*rob*); $^{**}P>0{\cdot}05$ versus KM-D; Fig. 2a]. The analyses of the deletion strains and the complemented mutants were investigated in independent experiments. The relative virulence of these strains is compared with the controls (KM-D and PC1012) in (a).

marA, *soxS* and *rob* were then restored individually in their original locations in the chromosome of PC1012. Complementation with single chromosomal copies of each transcription factor resulted in a strain that colonized the kidney to a similar extent as the wild-type host KM-D (compare PC1012 with PC1033, PC1037 and PC1038 in Fig. 2a). Thus, *marA*, *soxS* or *rob*, alone, is sufficient in restoring kidney colonization in the absence of the other two genes.

We next determined whether deletion of a single transcription factor affected kidney colonization. Deletion of *soxS* or *rob* produced an organism that exhibited a significant ($P<0{\cdot}05$) defect in kidney colonization (compare KM-D with PC1005 and PC1003, Fig. 2b). A statistically significant effect ($P>0{\cdot}05$), however, was not seen in the strain deleted for *marA* (compare KM-D with PC1001, Fig. 2b). In the case of PC1005 and PC1003, the colonization capacity of the host was fully restored when the single chromosomal copy of either *soxS* or *rob* was replaced in the respective single-deletion strains (compare KM-D with PC1035 and PC1040, Fig. 2b). Thus, while SoxS and Rob appear to be more critical than MarA for kidney colonization in KM-D, each restored kidney colonization in the triple mutant.

A series of single genetic deletions was constructed in *E. coli* C189, which is an authentic uropathogenic *E. coli* (UPEC) isolate obtained from a patient with cystitis (Rippere-Lampe *et al.*, 2001; Yamamoto *et al.*, 1995). Similar to the results observed with KM-D, deletion of the gene specifying either SoxS or Rob, but not MarA, in the C189 background, compromised the colonization capacity of the bacterium.

The median c.f.u. (g kidney)$^{-1}$ obtained following infection with either the single *soxS* or *rob* deletion mutant was 1, whereas a median of $1{\cdot}4 \times 10^4$ c.f.u. (g kidney)$^{-1}$ was recovered following infection with the *marA* mutant (data not shown), which was similar to the wild-type strain. A statistically significant difference ($P<0{\cdot}05$, using a non-parametric Wilcoxon method), however, was only achieved with the *soxS* mutant (data not shown).

These findings strongly suggest that the colonization defect seen in the mutants is associated with the absence of SoxS or Rob, and they suggest little, if any, role for MarA if either SoxS or Rob is present. That a detrimental effect following deletion of *marA* alone in either *E. coli* strain was not seen may reflect 'backup' activity of Sox and/or Rob for critical genes regulated by the transcription factors. However, the *marRAB* operon is induced during growth of UPEC in another mouse model of UTI (Snyder *et al.*, 2004).

To rule out that the genetic manipulations needed to produce the deletion strains might have a detrimental effect on the ability of the organism to colonize the murine kidneys, we investigated the colonization capacity of two wild-type strains that were obtained during the procedure used to produce the in-frame knockouts. The mutagenic process used in this study (Link *et al.*, 1997) resulted in a population of bacteria in which half could be wild-type, and the other half mutant. Two wild-type strains, PC wt Rob and PC wt MarA, were indistinguishable from C189-P2 in their ability to colonize the murine kidney ($2{\cdot}0 \times 10^4$ and $1{\cdot}2 \times 10^4$, versus $1{\cdot}6 \times 10^4$ median c.f.u. (g kidney)$^{-1}$, respectively; data not shown).

There is a limitation of this murine UTI model. Within the experiments, some mice maintained colonization with the deletion strain PC1012 (e.g. see Fig. 1b). Similar findings of 'outliers' have also been observed with *Proteus mirabilis* UreR mutants (Dattelbaum *et al.*, 2003), and when the model has been used to test antibiotics, including an aminoglycoside, a penicillin, a cephalosporin, or trimethoprim-sulfamethoxazole, to treat susceptible *E. coli* infection (Hvidberg *et al.*, 2000; Kerrn *et al.*, 2003; Schilling *et al.*, 2002). Complete sterilization of any portion (urine, bladders or kidneys) of the mouse urinary tract has not been achieved in this model using genetic or therapeutic means (Dattelbaum *et al.*, 2003; Hvidberg *et al.*, 2000; Kerrn *et al.*, 2003; Schilling *et al.*, 2002).

The use of relatively large (100 µl) volumes in intravesicular inoculations, i.e. directly into the bladder, can result in vesicoureteral reflux (VUR) in the mouse (Hopkins *et al.*, 1995). While some have used intravesicular inoculation (Brzuszkiewicz *et al.*, 2006; Hopkins *et al.*, 1998), others have relied on the use of catheters to introduce bacteria directly into the bladder via the urethra. We tried unsuccessfully to use catheters for intraurethral infections. We do not think that VUR complicates the interpretation of our results for the following reasons. The time course of colonization was followed out to a period of 11 days, and at this point the preponderance of the murine kidneys was colonized by wild-type bacteria (Fig. 1a). Any short-term effect that might be manifested by VUR would be negated during this extended period of time. Also, since the mutants were analysed in the same model, they served as controls: they reached and colonized the kidney for up to 3 days, but were unable to maintain colonization thereafter.

The presence of bacteria within an otherwise sterile mouse kidney, nevertheless, correlates well with an active infection (Hvidberg *et al.*, 2000). The model used in this study reports on the inability of *E. coli* deletion mutants to colonize the mouse kidney; colonization is a critical component required for most bacterial infections.

Genetic, biochemical and phenotypic characterization of *E. coli* strains

PCR was used to determine the presence of a number of genes known to be involved in the virulence of UPEC. We compared the results obtained using total DNA from C189-P2 and KM-D. The *aer* gene (specifying the aerobactin siderophore), or the *fimA* and *fimH* genes (encoding the type I fimbriae), were present in both of the clinical isolates (Table 2). The UPEC isolate C189-P2 contained many of the other genetic sequences sought (Table 2). The gene *afa* (encoding afimbrial adhesions) was not detected by PCR in C189-P2, KM-D or *E. coli* K-12 MG1655. C189-P2, as reported, also lacks *hly* (Yamamoto *et al.*, 1995); KM-D lacks *hly* and a number of genes specifying many of the known UPEC virulence factors (Table 2), but it colonized the mouse kidney to a similar extent as C189-P2. Moreover, both KM-D and C189-P2 showed a similar decrease in colonization in the mouse UTI model when either *soxS* or

Table 2. Virulence factors present, as determined using PCR, in strains included in this study

Gene products are specified in parentheses. The genes *afa* (afimbrial adhesions) and *hly* (haemolysin) were not found in either C189 or KM-D. The genes *fimA* and *fimH*, but not any of the other virulence-associated genes, were also present in *E. coli* K-12, a laboratory isolate. +, Present; −, not present.

Gene	Host	
	C189	KM-D
aer (aerobactin)	+	+
cnf (cytotoxic necrotizing factor)	+	−
fimA (type 1 pili)	+	+
fimH (type 1 pili)	+	+
papC (pyelonephritis-associated pili)	+	−
papE/F (pyelonephritis-associated pili)	+	−
papGII (pyelonephritis-associated pili)	+	−
sat (autotransporter toxin)	+	−
sfa (S fimbriae)	+	−
traT (serum resistance)	+	+

rob was deleted. The *traT* gene (specifying a protein involved in serum resistance; Sukupolvi & O'Connor, 1990), another *E. coli* virulence factor, was also detected by PCR in both KM-D and C189-P2 (Table 2).

Based on the results from PCR, *aer*, *fimA* and *fimH*, and *traT* seemed to be virulence candidates that were potentially affected by the deletions, since they were present in both KM-D and C189-P2. We initially ruled out *aer*, since we reasoned that affecting any one siderophore (if others were present) would not have such a dramatic effect on colonization *in vivo* (Torres *et al.*, 2001). A role for siderophore-related functions, e.g. IroN, however, has been established using *in vivo* competition experiments (to detect subtle changes in virulence) between wild-type and mutant bacteria in a mouse model of UTI (Russo *et al.*, 2002). The roles of the *fimA*, *fimH* and *traT* gene products were further investigated using a limited set of virulence assays (see below).

In order to assess the integrity of the bacterial outer membrane, the LPS content of KM-D, and its triple deletion mutant PC1012, was qualitatively examined using bacteria grown in LB or M9/glucose broth, or from cells isolated directly from LB agar plates (Westphal & Jann, 1965). No differences were seen in either the LPS core or the O-antigen between the two strains on silver-stained SDS-PAGE gels (data not shown). The integrity of the cell envelope was further tested by measuring susceptibility to SDS and crystal violet in a standard disk diffusion assay using drug-impregnated paper disks. Both KM-D and PC1012 were resistant to SDS, and exhibited the same zone of inhibition ($\sim$1 cm) when incubated in the presence of a disk containing crystal violet (data not shown).

KM-D, C189-P2, PC1012 and PC*rob* were tested for their susceptibility to active mouse serum, and their ability to grow in heat-inactivated mouse serum. No differences were seen (data not shown), suggesting that the expression of TraT is not involved in the colonization defect exhibited by PC1012 and PC*rob*.

The expression of type I pili was monitored qualitatively using mannose-sensitive agglutination of guinea pig red blood cells. Both C189-P2 and KM-D caused haemagglutination. This property was not seen with *E. coli* EP-1, which lacks type I fimbriae. The agglutination was abrogated by mannose, the natural substrate of type I fimbriae, indicating a specific effect of type I fimbriae (data not shown). The PC*rob*, *soxS* (double deletion) and PC1012 (triple deletion) mutants caused haemagglutination at levels similar to their wild-type parent strains (data not shown). In all instances (except for the EP-1 strain), increasing the bacterial concentration 10-fold did not result in additional haemagglutination, and reducing the bacterial concentration 10-fold largely eliminated haemagglutination for all isolates (data not shown). Thus, the expression of type I fimbriae does not appear to be dependent on the presence of MarA, SoxS or Rob.

Relative to wild-type organisms, the mutant strains produced in this study did not show any statistically significant differences in growth rate, cytotoxicity toward tissue culture cells, adherence and internalization in cell culture (Garrity-Ryan *et al.*, 2000), and induction of cytokine expression (Hedlund *et al.*, 1999) (data not shown).

Using a limited set of known virulence assays, we were unable to find a specific defect that would account for the lack of kidney colonization, or the difference between the *marA*-deletion strains and strains deleted of the other two transcription factors. Given the number of genes regulated by SoxS (Pomposiello & Demple, 2000; Pomposiello *et al.*, 2001) and Rob, the inability to find a defect in one particular virulence factor was not entirely unexpected. Since MarA, SoxS and Rob are not required for growth of the organism *in vitro*, it may simply be that a regulatory defect in many genes simultaneously has as much of an effect on growth *in vivo* as does the removal of a single critical virulence factor, e.g. type I fimbriae. For example, it is known that some of the genes regulated by MarA are involved in biofilm formation (Otto *et al.*, 2001), and this phenotype has been shown recently to be important for the pathogenesis of UTI in mice (Anderson *et al.*, 2003).

The antibiotic-resistance phenotype observed in strains overexpressing MarA, SoxS and Rob depends largely on the AcrAB–TolC efflux system (Alekshun & Levy, 1999). Although multidrug-resistance efflux systems have been shown to affect virulence (Piddock, 2006), a connection between MarA, SoxS and Rob, and efflux in the present work, is not clear. Moreover, since the wild-type and deletion strains grew equally well on MacConkey agar, a difference in susceptibility to bile salts was also ruled out, as was a major defect in AcrAB–TolC expression *in vitro*.

Concluding remarks

While a number of phenotypes have been assigned to MarA, SoxS and Rob transcription factors *in vitro*, e.g. resistance to antibiotics, household disinfectants and oxidative stress agents (Alekshun & Levy, 1999), this is one of the first reports to demonstrate an effect of these proteins *in vivo*. That a family of bacterial transcription factors, which are not required for growth *in vitro*, has been shown to be necessary for persistence in a mouse model of UTI suggests that the factors might be exploited as new therapeutic targets. Small-molecule inhibitors of these transcription factors (Alekshun & Levy, 2005) can be designed to be used alone to prevent infection, or in conjunction with an antibiotic to help treat an infection. A recent study has extended this concept to *V. cholerae* infection (Hung *et al.*, 2005).

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